

AD _____

Award Number: W81XWH-08-1-0456

TITLE: In utero influences, breast stem cells, and breast cancer risk factors

PRINCIPAL INVESTIGATOR: Chung-Cheng Hsieh

CONTRACTING ORGANIZATION: University of Massachusetts Medical Center
Worcester, MA 01655

REPORT DATE: August 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 31/08/2009		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 August 2008 – 31 July 2009	
4. TITLE AND SUBTITLE In utero influences, breast stem cells, and breast cancer risk factors				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0456	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Chung-Cheng Hsieh Email: Chung.Hsieh@umassmed.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) * University of Massachusetts Medical Center Worcester, MA 01605				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Breast density can be assessed by whole mount carmine alum staining of the fourth inguinal mammary glands according to the method reported by Brisken et al (2002) and counting the number of terminal end buds. Mammary glands of pregnant C57Bl/6J mice were found to have significantly more terminal end buds than virgin mice. Flow cytometric analyses of dissociated cells from mammary glands clearly identified a sub-population of cells that are CD49f ⁺ and CD24 ⁺ , putative markers of mammary stem/progenitor cells.					
15. SUBJECT TERMS Breast stem cells, breast density, insulin-like growth factor-1, perinatal factors, cancer risk factors					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	5
Reportable Outcomes.....	5
Conclusion.....	6

INTRODUCTION

The objective of this proposed study is to understand the biological determinants of mammographic density, a strong predictor of human breast cancer risk. Our hypothesis is that the *in utero* levels of mitogens, such as IGF-1, drive an increased number of breast stem/progenitor cells in the mammary gland, which in turn results in a more extensive epithelial mammary tree formation, i.e., an increase in mammographic density. Since breast stem/progenitor cells are the cell population within the mammary gland that is presumed to be susceptible to malignant transformations, dense breast with elevated levels of stem/progenitor cells, should have a higher risk of becoming malignant. The study hopes to correlate *in utero* levels of IGF-1 administered to the mother with the birth weight, breast/mammographic density and number of breast stem/progenitor cells of the offspring.

BODY

Timetable of research accomplishments as outlined in the Statement of Work:

- Obtain approval for animal research; develop and validate microparticle delivery vehicle generation. (Months 0-3)
- Obtain IGF-1^{m/m} and control mice and begin breeding. (Months 3-6)
- Perform preliminary studies to quantitate and validate breast stem/progenitor FACS assay and breast density assay. (Months 3-6)
- Perform experiments on effect of *in utero* IGF-1 treatment on breast stem/progenitor pools and breast density using cohorts of bred mice. (Months 8-32)
- Compile data, perform statistical analysis and write manuscript. (Months 32-36)

Progress report:

Since the launching of the project, we have obtained approvals for animal research from the Institutional Animal Care and Use Committee (IACUC), University of Massachusetts Medical School (UMMS) in May 2008 and from the Animal Care and Use Review Office (ACURO), Department of The Army in June 2008 (Task a).

We performed preliminary studies to quantitate and validate breast density using histological assays, and by *in vivo* and explant imaging (Task c). Histologically, we stained mammary glands from virgin and pregnant C57Bl/6J mice using whole-mount staining protocols as reported by Nandi (1958), Fata et al (1999) and Brisken et al (2002). Although all three methods gave good staining of mouse mammary glands as visualized under a microscope, with a greater number of terminal end buds from mammary glands of pregnant mice (**mean \pm standard error of the mean (SEM) of terminal end bud-like structure counts of 626 ± 25.4 , $n = 4$**) than from virgins (**5.5 ± 0.5 , $n = 2$**), the method as reported by Brisken gave the best staining of terminal end buds (**Fig 1**). We improved on the method by cover-slipping the slides using Permount (Fisher Scientific) whereby the stained mammary glands are preserved indefinitely and can be viewed microscopically for analysis at any time. We also explored whether breast/mammographic density can be measured radiographically using a Nanospect/CT *in vivo* animal imager, available at the Small Animal Imaging Core Facility, UMMS. To do this, we wrote a separate animal protocol and obtained approval from IACUC, UMMS in May 2009. Although the device could image the mammary glands both *in vivo* and as explants, it could not detect the difference in radiographic density of mammary glands between virgin and pregnant mice.

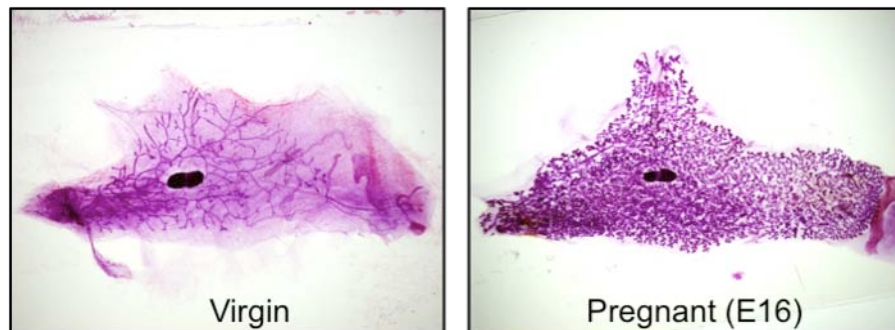


Fig 1. Whole mount fourth inguinal mammary glands of C57Bl/6J mice stained with carmine alum solution, mounted and cover-slipped on glass slides using Permount showed that a mammary gland from a virgin mouse (left panel) is “less dense” than that of a E16 pregnant gland (right panel); both glands are from 8 week-old mice.

We performed preliminary studies to quantitate and validate stem/progenitor using flow cytometric assay (Task c). Using protocols adapted from StemCell Technologies Inc. (Vancouver, Canada) and Stingl et al (2006), we successfully detected a sub-population of cells from mammary glands that are CD49f⁺ and CD24⁺, putative markers of mammary stem/progenitor cells. **In mammary glands of wild type C57Bl/6J control mice, the CD49f⁺/CD24⁺ population ranged from 0.42 to 1.5% of total cells, with a mean \pm SEM value of $1.06 \pm 0.23\%$ (n = 4) (Fig 2).** We are presently investigating whether we can also detect other putative markers of mammary stem/progenitor cells, such as CD24⁺/CD29⁺ (Shackleton et al, 2006) and CD24⁺/CD29⁺/CD49f⁺ (Visvader and Lindeman, 2006).

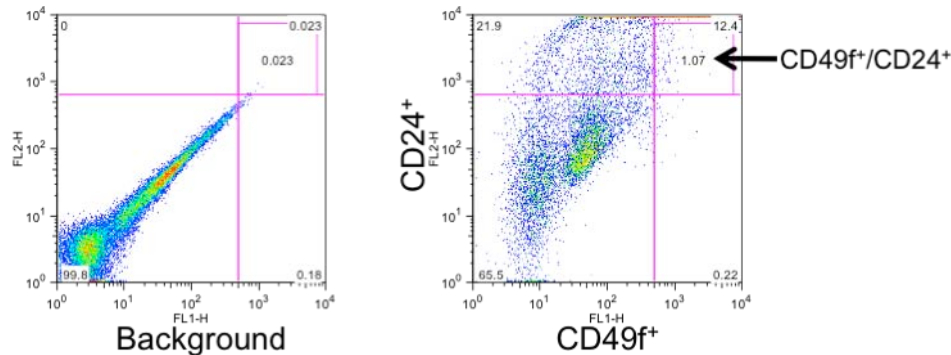


Fig 2. Flow cytometric dot plots showing the identification of a population of CD49f⁺/CD24⁺ cells in the upper right quadrant (arrow) from C57Bl/6J dissociated mammary cells.

Homozygous *Igf1^{m/m}* mice are being prepared for the project (Task b). While this is being carried out, investigations are being carried out using wild type mice (Task b and d). We are performing studies using wild type C57Bl/6J mice to determine the optimal dose of IGF-1 that can confer an *in utero* effect on the birth weights and mammary stem/progenitor cells of the offspring. We found that the original proposal of delivering IGF-1 microparticles trans-placentally into pregnant mice to be cumbersome and caused unnecessary trauma to the mice. We now propose to administer IGF-1 into pregnant mice via daily intra-peritoneal (ip) injections on embryonic days (E) 10 to 16, inclusive. We have written a minor amendment for this proposed change that was approved by IACUC, UMMS in June 2009. We have investigated 3 concentrations of IGF-1 (5 µg/kg, 10 µg/kg and 15 µg/kg of maternal body weight) with vehicle for controls and the results are being analyzed.

Literature cited:

1. Briskin C, Socolovsky M, Lodish HF, Weinberg R. The signaling domain of the erythropoietin receptor rescues prolactin receptor-mutant mammary epithelium. PNAS 2002;99:14241-5.
2. Fata JE, Leco KJ, Moorehead RA, Martin DC, Khokha R. Timp-1 is important for epithelial proliferation and branching morphogenesis during mouse mammary gland development. Dev Biol 1999;211:238-54.
3. Nandi S. Endocrine control of a mammary-gland development and function in the C3H/He Crgl mouse. J Natl Cancer Inst 1958;1039-63.
4. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat M-L, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. Nature 2006;439:84-8.
5. Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li H, Eaves CJ. Purification and unique properties of mammary epithelial stem cells. Nature 2006;439:993-7.
6. Visvader JE, Lindeman GJ. Mammary stem cells and mammaryogenesis. Cancer Res 2006;66:9798-801.

KEY RESEARCH ACCOMPLISHMENTS

- Breast/mammographic density was successfully assessed by whole mount carmine alum staining of the fourth inguinal mammary glands.
- Coverslipping using Permount of whole mount carmine alum stained mammary glands preserved the histological staining indefinitely.
- A putative sub-population of mammary stem/progenitor cells that were CD49f⁺ and CD24⁺ was successfully identified from dissociated cells of mammary glands.

REPORTABLE OUTCOMES

- By assessing breast density by whole mount carmine alum staining of the fourth inguinal mammary glands, mammary glands of pregnant C57Bl/6J mice were found to have significantly more terminal end buds than virgin mice.

- Flow cytometric analyses of dissociated cells from mouse mammary glands clearly identified a sub-population of cells that are CD49f⁺ and CD24⁺, putative markers of mammary stem/progenitor cells.

CONCLUSIONS

This project is progressing according to plan.